

PATENT APPLICATION**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re the Application of

Kelvin G.M. BROCKBANK et al.

Group Art Unit: 1651

Application No.: 10/670,724

Examiner: S. SAUCIER

Filed: September 26, 2003

Docket No.: 113024

For: METHOD FOR TREATMENT OF CELLULAR MATERIALS WITH SUGARS
PRIOR TO PRESERVATION

DECLARATION UNDER 37 C.F.R. §1.131

I, Kelvin G. M. BROCKBANK, a citizen of the United Kingdom, hereby declare and state:

1. This Declaration is submitted as evidence that the subject matter claimed in the above-identified application was invented prior to July 26, 2002, the earliest U.S. filing date of U.S. Patent Publication No. 2005/027710 ("Toner").
2. I am a named inventor in the above-captioned patent application.
3. I am one of the authors of the attached Invention Proposal ("IP"), dated prior to July 26, 2002, a true copy of which appears as Exhibit A attached to this Declaration.
4. Exhibit A describes a method for preserving living cellular material, comprising incubating the cellular material in a culture medium containing at least one sugar for at least three hours; and after the incubation, subjecting the cellular material to a preservation protocol, wherein the culture medium contains sugar, and wherein the at least one sugar comprises trehalose. Exhibit A further describes a method for preparing living cellular material for preservation, comprising incubating the cellular material in a culture medium containing at least one polysaccharide for at least three hours, wherein the culture

Application No. 10/670,724

medium contains polysaccharide, and wherein the at least one polysaccharide comprises trehalose. In particular, the specifics of the method for preserving living cellular material and a method for preparing living cellular material for preservation disclosed in the above-identified application are described on pages 2 through 4 of Exhibit A.

5. The remainder of the IP sets forth further details of the design. For example, details of the incubation time, sugars, preservation protocol, viability, etc., are described on pages 2 through 4 of the IP.

6. The invention described in Exhibit A may thus be summarized as follows:

(a) a method for preserving living cellular material or a method of preparing living cellular material, comprising incubating the cellular material in a culture medium containing at least one sugar for at least three hours; and

(b) if preserving the living cellular material, after the incubation, subjecting the cellular material to a preservation protocol,

(c) wherein the culture medium contains sugar, and

(d) wherein the at least one sugar comprises trehalose.

7. Exhibit A describes an invention conceived and reduced to practice prior to July 26, 2002. This invention is claimed in the above-identified application.

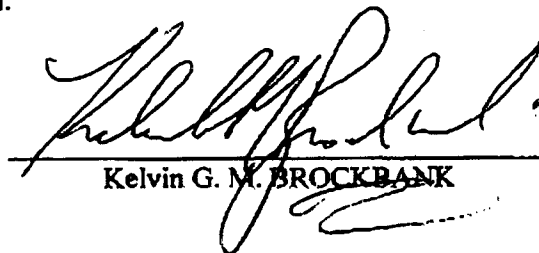
8. Prior to July 26, 2002, I and/or those under my direct supervision and control, carried out a reduction to practice of the invention described in Exhibit A and thereby provided the methods described in paragraph 4-7 herein.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18

Application No. 10/670,724

of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Date: July 13th 2006


Kelvin G. M. BROCKBANK

METHOD FOR TREATMENT OF CELLULAR MATERIALS WITH SUGARS

PRIOR TO PRESERVATION

Field of the invention: The invention relates to the field of cell, tissue and organ preservation. More specifically, the invention relates to a method for treatment of cellular materials with sugars prior to preservation that results in enhanced cell survival post-preservation. This is particularly important because the sugars, trehalose and sucrose, are not cytotoxic to cells and may not have to be removed before living, natural or man-made biological materials are transplanted into humans or animals.

Background of the invention: Conventional approaches to cryopreservation that provided the cornerstone of isolated cell storage have not been successfully extrapolated to more complex natural, or engineered, multicellular tissues. Tissues are much more than simple aggregates of various cell types; they have a highly organized, often complex, structure that influences their response to freezing and thawing. The formation of extracellular ice, in particular, which is generally regarded as innocuous for cells in suspension, is known to be a hazard to structured tissues and organs. Cryopreservation is a complex process of coupled heat and mass transfer generally executed under non-equilibrium conditions. Advances in the field were modest until the cryoprotective properties of glycerol and dimethyl sulfoxide (DMSO) were discovered in the mid 1900's.^{1,2} Many other cryoprotective agents (CPAs) have since been identified. Combinations of CPAs may result in additive or synergistic enhancement of cell survival by minimization of intracellular ice during freezing.³

Restriction of the amount and size of extracellular ice crystal formation during cryopreservation can be achieved by using high concentrations of CPAs that promote amorphous solidification, known as vitrification, rather than crystallization.⁴ Vitrification is a relatively well understood physical process, but its application to the preservation of biological systems has not been without problems, since the high concentrations of CPAs necessary to facilitate vitrification are potentially toxic. To limit toxic effects it is necessary to use the least toxic CPAs at the lowest concentrations that will still permit glass formation (at cooling rates that are practical for bulky mammalian tissues).⁴ Comparison of the effects of vitrification and conventional frozen cryopreservation upon venous contractility demonstrated that vitrification is superior to conventional cryopreservation methods.⁴ Vitrification has more recently been used effectively for a variety of other tissues including myocardium, skin, and articular cartilage.

However, both conventional freezing and vitrification approaches to preservation have limitations. First, both of these technologies require low temperature storage and transportation conditions. Neither can be stored above their glass transition temperature for very long without significant risk of product damage due to ice formation and growth. Both technologies require competent technical support during the rewarming and CPA elution phase prior to product utilization. This is possible in a high technology surgical operating theater but not in a doctor's outpatient office or in third world environments. In contrast, theoretically, a dry product would

have none of these issues because it should be stable at room temperature and rehydration should be feasible in a sterile packaging system.

Drying and vitrification have previously been combined for matrix preservation of cardiovascular and skin tissues, but not for live cell preservation in tissues or engineered products. Nature, however, has developed a wide variety of organisms and animals that tolerate dehydration stress by a spectrum of physiological and genetic adaptation mechanisms. Among these adaptive processes, the accumulation of large amounts of disaccharides, especially trehalose and sucrose, is especially noteworthy in almost all anhydrobiotic organisms including plant seeds, bacteria, insects, yeast, brine shrimp, fungi and their spores, cysts of certain crustaceans, and some soil-dwelling animals.⁵⁻⁷ The protective effects of trehalose and sucrose may be classified under two general mechanisms: (1) "the water replacement hypothesis" or stabilization of biological membranes and proteins by direct interaction of sugars with polar residues through hydrogen bonding, and (2) stable glass formation (vitrification) by sugars in the dry state.

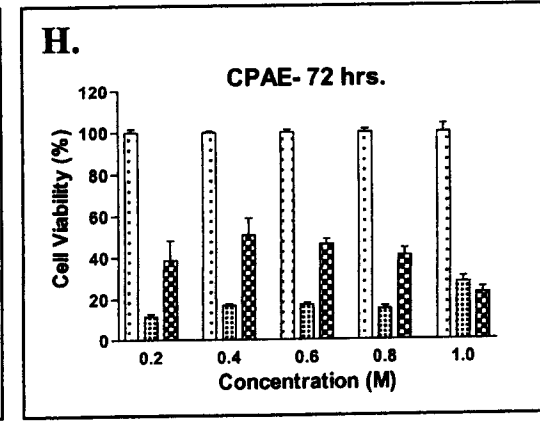
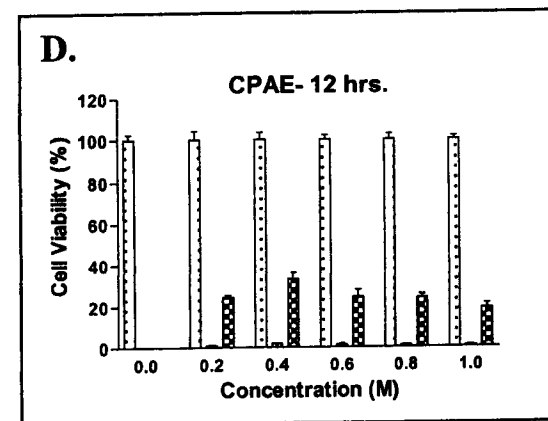
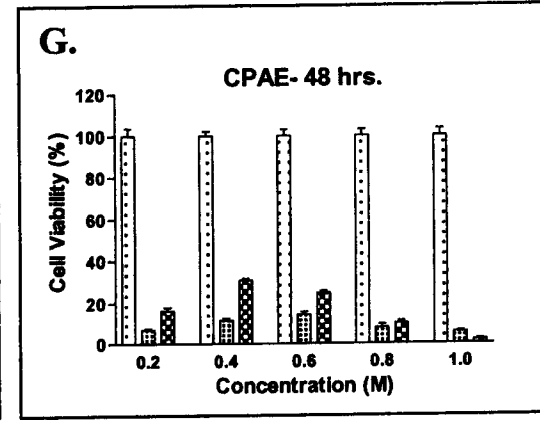
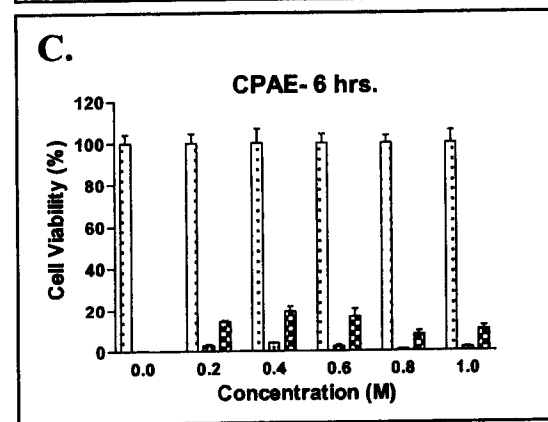
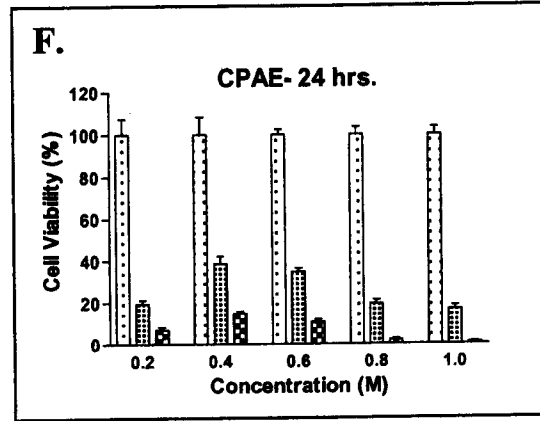
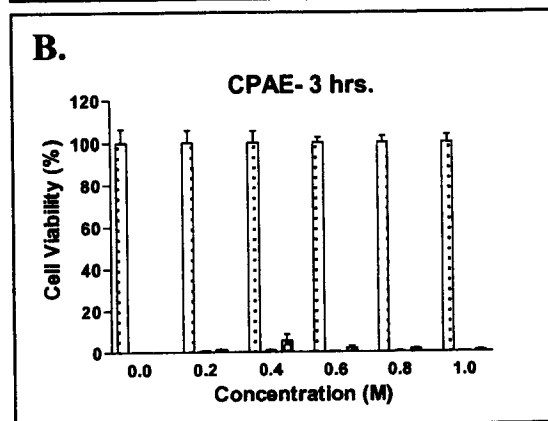
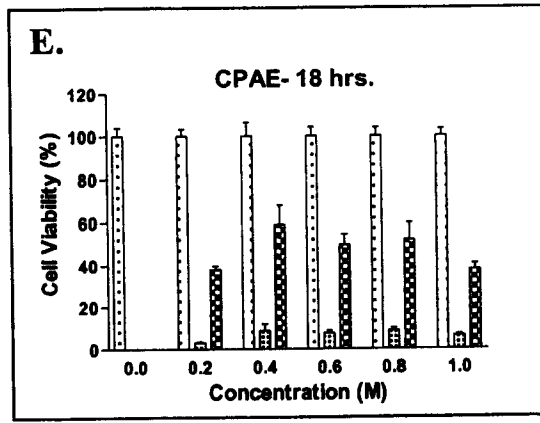
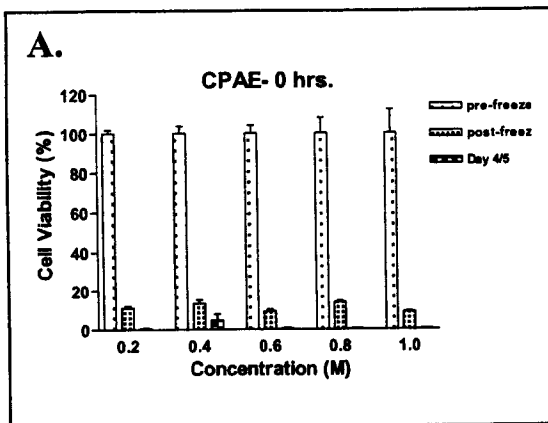
The stabilizing effect of these sugars has also been shown in a number of model systems, including liposomes, membranes, viral particles, and proteins during dry storage at ambient temperatures.⁸⁻¹⁰ On the other hand, the use of these sugars in mammalian cells has been somewhat limited, mainly because mammalian cell membranes are believed to be impermeable to disaccharides or larger sugars.¹¹ For sugars to be effective they need to be present both on the inside and the outside of the cell membrane. Several methods have been developed for loading of sugars in living cells. Recently, a novel, genetically-modified, metal-actuated switchable membrane pore has been used to reversibly permeabilize mammalian cells to sugars with significant post-cryopreservation and, to lesser extent, drying cell survival.¹² Other permeation technologies, that have been considered for placing sugars in cells include use of pressure, electroporation, microinjection, and thermal and osmotic shock. The expression of sucrose and trehalose synthase genes and transporters has also been considered as means for delivery of sugars into cells. Introduction of trehalose into human pancreatic islet cells during a cell membrane thermotropic lipid-phase transition, prior to freezing and in the presence of a mixture of 2M DMSO and trehalose, resulted in previously unattainable cell survival rates.¹³ This method depends upon suspension of cells in a trehalose solution and either cooling or warming the solution through the thermotropic transition of the cells.¹⁴ Human fibroblast transfection with *E. coli* genes expressing trehalose resulted in retention of viability after drying for up to five days.¹⁵

Clearly, the potential value of sugars, such as trehalose and sucrose, has been recognized for many years. We have been working on the metal-actuated switchable membrane pore system to reversibly permeabilize mammalian cells to sugars. In the course of these studies, short-term preincubation with sugars, prior to poration, resulted in improved experimental outcomes. These observations led to the testing of longer incubation times without subsequent poration as controls for possible sugar effects. These experiments led to the unanticipated discovery that incubation of cellular materials under physiological conditions in the presence of low concentrations of sugars resulted in increased cell survival without any need for the metal-actuated switchable membrane pore.

Definitions: Preservation - a process for provision of shelf life to a cell containing, living biological material. Preservation processes include cryopreservation by freezing and vitrification and anhydrobiotic preservation by either freeze-drying or dessication. Living biological materials includes all materials natural or man made with cellular components.

Summary of the invention: The present application provides a method for pre-treatment of cellular materials with sugars that enhances the ability of said cellular materials to survive a subsequent preservation procedure. Incubation of cellular materials in sugars under physiological conditions for short periods of time (less than 3 hours), with or without simple addition of extracellular sugars just prior to cell preservation, results in few, if any, cells surviving preservation procedures. However, we have discovered that *prolonged* incubation with sugars under physiological conditions (greater than 3 hours) prior to preservation results in cell survival under conditions that would otherwise have resulted in minimal, if any, cell survival.

Method: A bovine pulmonary artery endothelial cell line, CPAE, was used for these experiments. Cells were plated the night before in 96-well microtiter plates at 20,000 cells/well, then exposed to 0.2M trehalose in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C for 0, 3, 6, 12, 18, 24, 48 and 72 hours. Following exposure and prior to freezing, cell viability was determined using the non-invasive metabolic indicator alamarBlue (Trek Diagnostics). A volume of 20 µl was added to cells in 200 µl of DMEM(10%FCS) and the plate was allowed to incubate at 37 °C for three hours. The plate was read using a fluorescent microplate reader (Molecular Dynamics) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The alamarBlue assay was chosen because it is non-toxic and can be used to assess the viability of the cells without damage. Thus, we could check cell viability and then immediately freeze the cells in the plate. Cells were subsequently placed in 0-1.0M trehalose in DMEM (50 µl) and immediately cryopreserved using a controlled-rate freezer at -1.0°C/min. The following day, the cells were thawed by incubation for 30 minutes at -20°C, followed by rapid thawing at 37°C. The trehalose was diluted with 150 µl of DMEM (10% fetal calf serum, FCS) and the cells were left for one hour at 37°C. Cell viability was then reassessed using alamarBlue by incubation for three hours. Cell viability was also assessed on day four or five post-thaw.



Claims: Our first reaction is that the claims should focus on "A method for treatment of living biological materials (cells, tissues and organs) that enhances (increases, improves?) survival of cells that are subsequently subjected to preservation protocols." Essentially we have discovered that periods of incubation under tissue culture conditions in the presence of sugars (disaccharides, trehalose effect demonstrated to date) for periods greater than three hours results in enhanced post-preservation cell survival.

In our first experiments we have used 0.2 M trehalose incubation with 0.2-1.0 M of extracellular trehalose being added at the time of preservation, all extracellular concentrations provide cell survival benefits. Neither incubation alone nor extracellular sugars alone conferred any cell survival.

Experiments are in progress to assess some other opportunities including:

- 1) We anticipate that addition of other cryoprotectants either with or without extracellular sugars will result in enhanced cell survival. We will want to incorporate the list of reagents we have used in prior cryopreservation patents in a dependant claim.
- 2) It is also possible that modification of culture conditions may result in further improvements in cell survival post-sugar incubation.
- 3) It is possible that other sugar concentrations or nutrient conditions may result in positive effects after less than three hours of incubation.

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